Using the ykkCD Riboswitch Aptamer as a Trojan-Horse Tetracycline Delivery Vehicle in Nanomedicinal Applications

An Honors Thesis (HONR 499)

By

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Abstract

Gold nanoparticles (AuNPs) can be functionalized with a wide variety of molecules through electrostatic, hydrophobic, or thiol-gold affinity interactions. AuNP conjugates combine the unique properties and functions of both the nanoparticle and the additional molecules. The ykkCD riboswitch serves as a regulator for a multidrug-resistance efflux pump. This riboswitch was first discovered in *B. subtilis* and specifically recognizes tetracycline family of antibiotics.

Our research is focused on attaching the riboswitch aptamer to well-characterized AuNPs and demonstrating that ykkCD remains functional following attachment. Future applications of this research include the delivery of tetracycline to resistant bacteria.

Acknowledgements

I would like to thank Dr. Timea Gerczei for advising me through this project. Her help, both in and out of the lab, has made this thesis possible. I would also like to acknowledge Dr. Emil Khisamutdinov, Kaela Miller, and the staff of the Ball State Chemistry department for helping at various times throughout the research process. Finally, I would like to thank Dr. Paul Coan for organizing the CRISP program, which provided an opportunity for me to start this project during the summer.

Process Analysis Essay

For my Honors thesis project, I performed research investigating the structure and function of the ykkCD riboswitch and of gold nanoparticles. The ykkCD riboswitch is a regulator for a multidrug-resistance efflux pump, which means that it allows certain bacteria to resist the effects of antibiotics. Antibiotic resistance is a growing problem in the healthcare field; it causes many deaths and creates millions of dollars of extra healthcare spending. In order to reduce the effects of antibiotic resistance, it is critical for researchers to learn more about the ways bacteria defend against drugs and toxins. This project provides new information on the topic and the results of this investigation will help prevent the effects of resistant bacteria.

The research presented in this project spans multiple semesters and represents numerous hours of lab work. The project involves techniques from both biotechnology and nanotechnology. The biotechnology portion of this project included synthesizing, purifying, and analyzing the DNA and RNA samples. The nanotechnology aspect of the project included synthesizing and purifying gold nanoparticles. These different fields came together in this project. This research process was helpful in developing technical skills that will be useful in my graduate study. I also think that this hands-on approach is a good way for me to learn about complex scientific methods. This project was challenging because it contained many difficult concepts and procedures, but through hard work I was able to complete this thesis. This project is meaningful because it makes a contribution to the global knowledge base of information regarding antibiotic resistance. I hope this work will be used by other researchers in the future to prevent the spread and the effects of antibiotic resistance.

Introduction

RNA Nanotechnology

Nanotechnology is the creation and application of materials on a nanometer scale. Applications of nanotechnology are seen in electronics, alternative fuel development and medicine. Historically, DNA and proteins have been most extensively studied for applications in medical nanotechnology. Within the last ten years, RNA has become a viable alternative material for nanoconstruction. RNA has become popular in research because it "can be designed and manipulated with a level of simplicity characteristic of DNA, meanwhile displaying flexibility in structure and diversity in function similar to that of proteins" (Guo 2).

In addition to its simultaneous diversity and simplicity, RNA provides many other advantages. RNA can self-assemble in a highly controlled and programmable manner. Self-assembly is a spontaneous process where a pre-existing sequence of nucleotides can form an organized structure consisting of a network of noncovalent interactions (Bui 7). RNA self-assembly is programmable and tertiary structure formation can be altered through the addition of ionic salts. RNA can also be produced easily and in large quantities. RNA is useful in biological applications because it can pass through the nuclear membrane of cells and regulated biological pathways.

For RNA to be a useful therapeutic tool, it can interact with nanoparticles to increase stability and gain the ability to enter human cells and tissues. Free, unmodified RNA is extremely unstable and is quickly degraded by nucleases when delivered into mammalian cells. This project focuses on using gold nanoparticles to act as a

supporting structure for the RNA so the macromolecule can be used in human tissues. Gold nanoparticle/RNA conjugates bring together the unique properties and functions of both materials (Figure 1). Conjugates can be easily created through the affinity interactions between the positively charged gold and negatively charged thiolated biomolecules. This attachment method is preferred because it leaves the native structure of the RNA intact. This allows the activity, selectivity, and specificity of the RNA structure to remain unaffected.

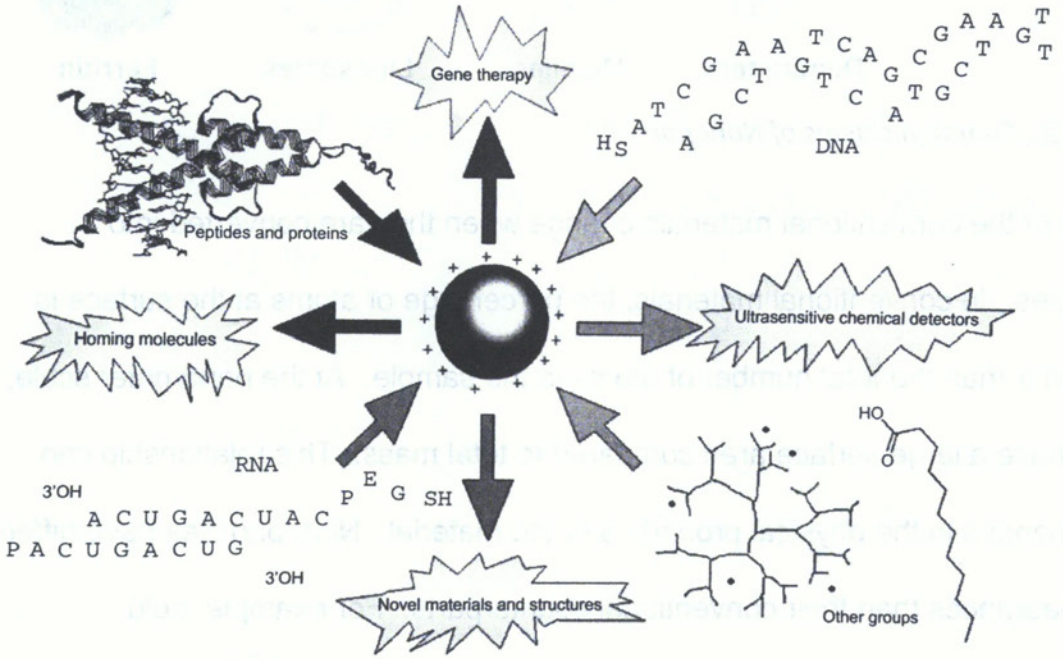

Figure 1 - Graphical abstract designed to highlight various applications of gold nanoparticles with different kinds of macromolecule conjugates - adopted from DeLong et al

Nanoparticles

Nanoparticles are particles of any shape with dimensions between 10^{-9} and 10^{-7} m and can form from many different materials. Categories of nanoparticles include carbon nanotubules, dendrimers, and metallic nanoparticles (Medina et al 553). This

project focuses on metallic nanoparticles, specifically, functionalized gold nanoparticles (Figure 2).

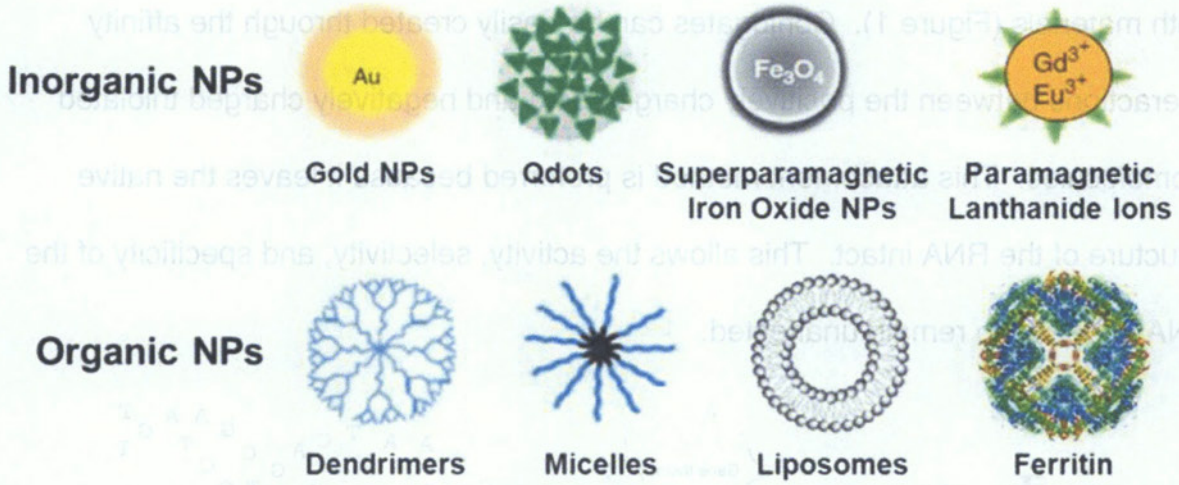

Figure 2 - Common classes of Nanoparticles

Properties of the conventional materials change when they are converted into nanoparticles. In conventional materials, the percentage of atoms at the surface is much smaller than the total number of atoms in the sample. At the nanometer scale, materials have a large surface area compared to total mass. This relationship can result in changes in the physical properties of the material. Nanoparticles have different visual appearances than their conventional counterparts. For example, gold nanoparticles appear a deep red color in solution, instead of gold. Other physical properties, such as malleability or ductility also change on the nanometer scale. These physical changes allow nanoparticles to be used in applications where conventional materials are ineffective. This project utilizes the nanoscale properties of gold to create functional conjugates of nanoparticles and RNA.

After the attachment of biological molecules, the functionalized particles can be used in gene therapy, as homing molecules, chemical detectors, or to create novel

materials and structures. For example, conjugates formed with RNA can be used to regulate gene expression. Conjugates formed with aptamers can be used to target a nanoparticle to a given cell type.

Riboswitches

Riboswitches are highly conserved sections of mRNA that can bind to small molecular ligands to regulate gene expression without the help of protein factors. Binding to the ligand induces structural changes in the riboswitch, which then controls expression of genes. This can either attenuate or upregulate transcription and translation, depending on the type of riboswitch. The general structure of a riboswitch consists of three components: aptamer, expression platform, and switching sequence. The aptamer is responsible for ligand binding. The expression platform is directly involved in altering transcription and translation of a downstream gene. The switching sequence lies between the aptamer and expression platform and can bind to both other components. While the general structure of a riboswitch is similar in all varieties, there is a high degree of diversity in the possible size and complexity. In addition to the wide range of structures, there are many different types of ligands that riboswitches can interact with. Currently, 20 different classes of riboswitches have been identified with specificity to a group of ligands (Figure 3). There are other riboswitch-like genetic control elements that lack a validated ligand, and this group is referred to as "orphan riboswitches."

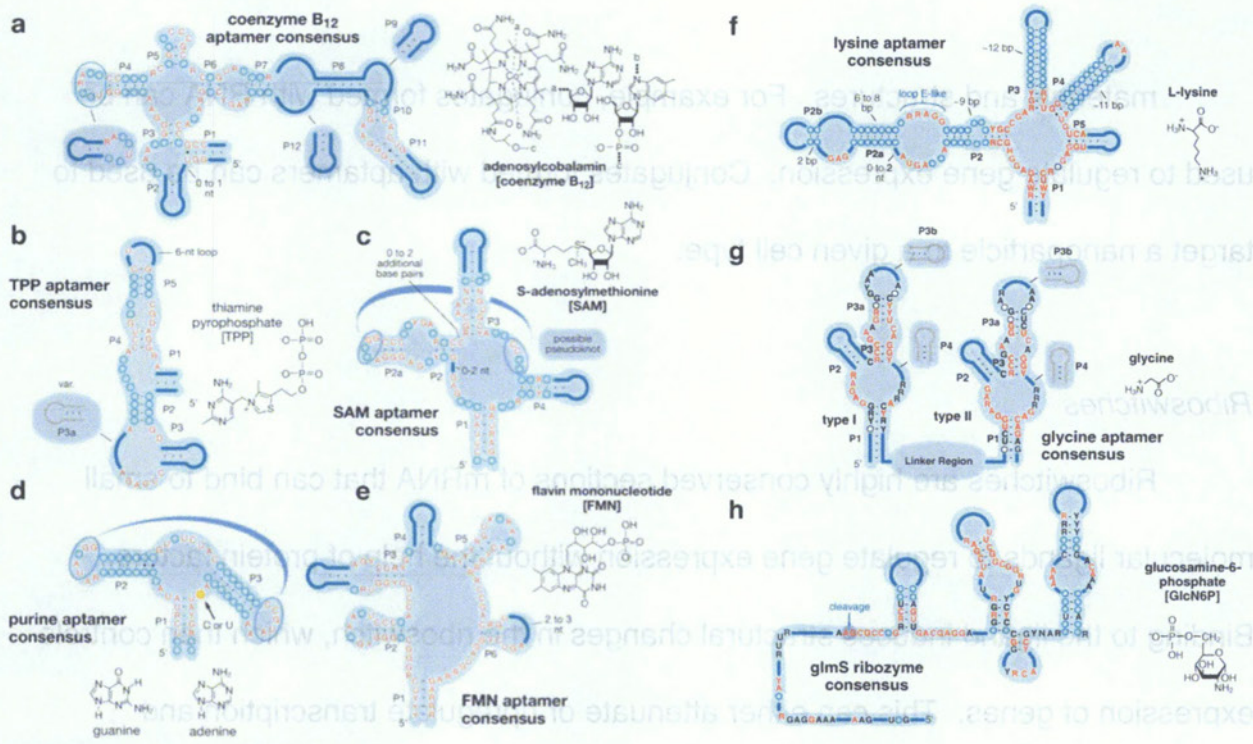

Figure 3 - Classes of riboswitches

This project focuses on the ykkCD riboswitch, which is a type of orphan riboswitch that was first discovered in *B. subtilis*. ykkCD appears to be a transcriptional ON-switch that regulates the expression of a multidrug-resistance efflux pump (ykkCD pump). This riboswitch specifically recognizes the tetracycline family of antibiotics. Ligand binding unfolds the terminator stem and permits the expression of the ykkCD pump, which expels the toxins from the cell. ykkCD was chosen for study because it has numerous innovative practical applications in the area of nanotechnology as a highly sensitive tet sensing module or a tet-responsive regulatory switch.

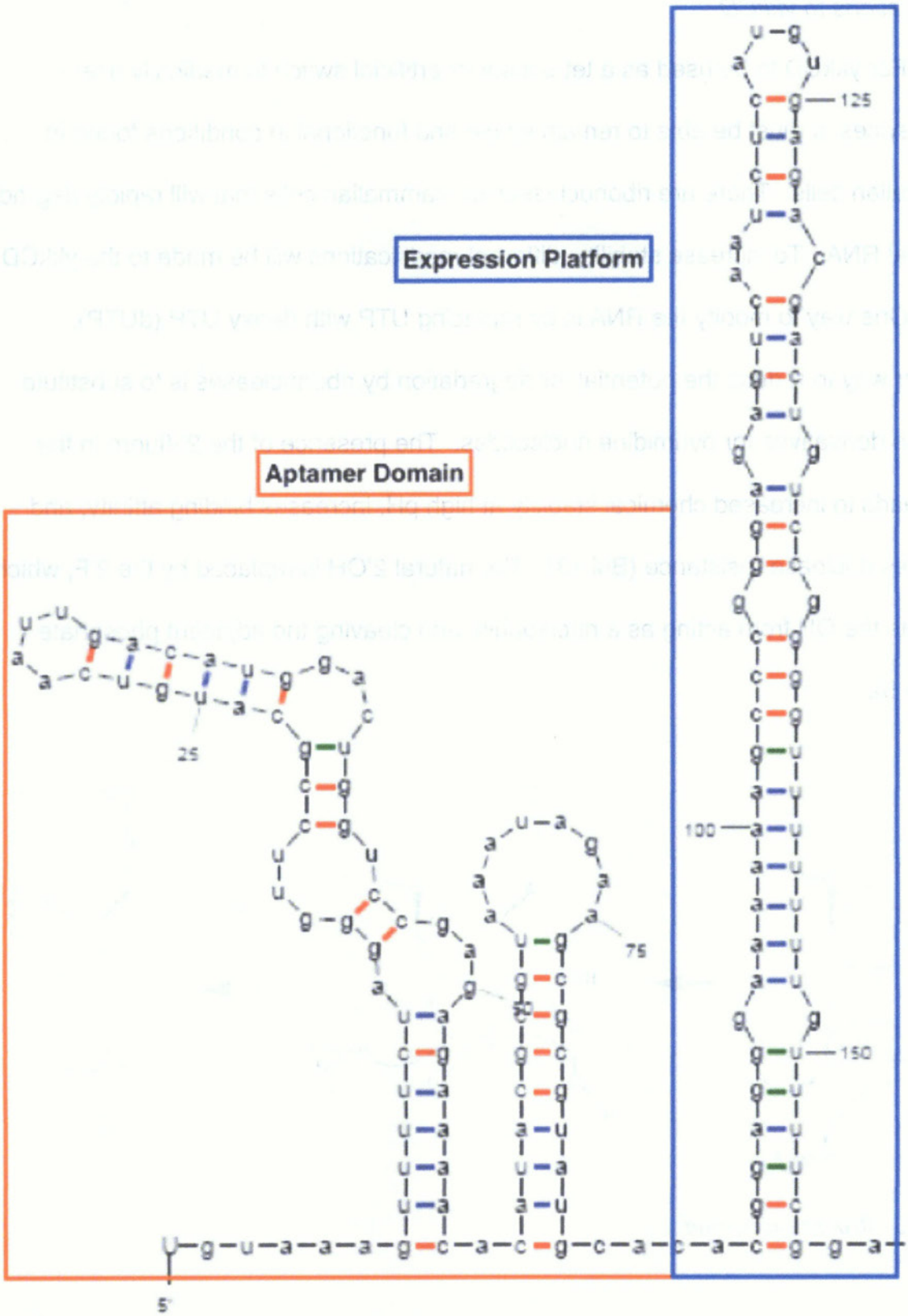

Figure 4 – Predicted Secondary Structure of ykkCD Riboswitch

Modifications to ykkCD

For ykkCD to be used as a tet-sensor or artificial switch in medically useful nanodevices, it must be able to remain stable and functional in conditions found in mammalian cells. There are ribonucleases in mammalian cells that will rapidly degrade bacterial RNA. To increase stability, different modifications will be made to the ykkCD RNA. One way to modify the RNA is by replacing UTP with deoxy UTP (dUTP). Another way to reduce the potential for degradation by ribonucleases is to substitute 2'Fluoro derivatives for pyrimidine nucleotides. The presence of the 2'-fluoro in the RNA leads to increased chemical stability at high pH, increases binding affinity, and improves nuclease resistance (Bui 13). The natural 2'OH is replaced by the 2'F, which prevents the OH from acting as a nucleophile and cleaving the adjacent phosphate (Figure 5).

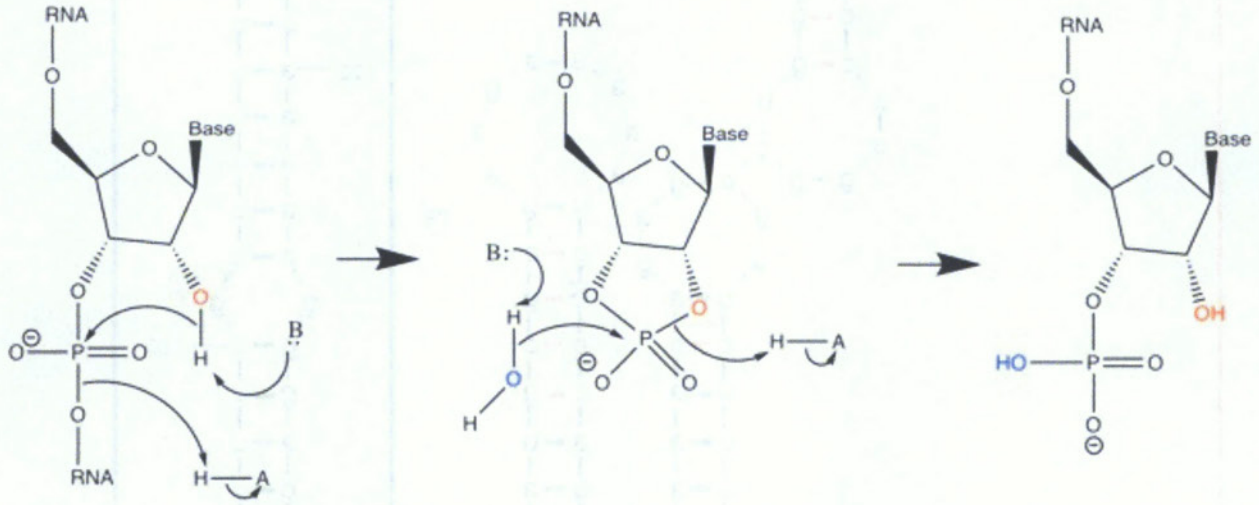

Figure 5 - RNA self-cleavage

This self-cleaving results in degradation of the RNA. Substitution of the of 2'-fluoro in the RNA structure does not significantly affect the conformation of the sugar ring, allowing the RNA to function the same as the natural version (Figure 6 & 7).

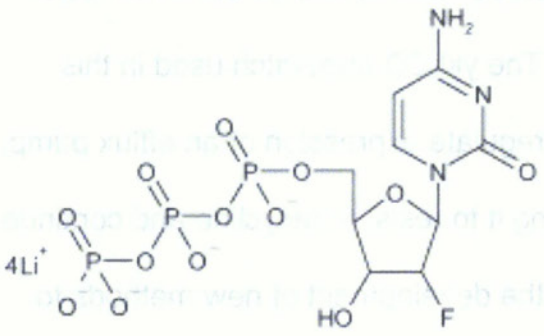

Figure 6 - Structure of 2'F-dCTP

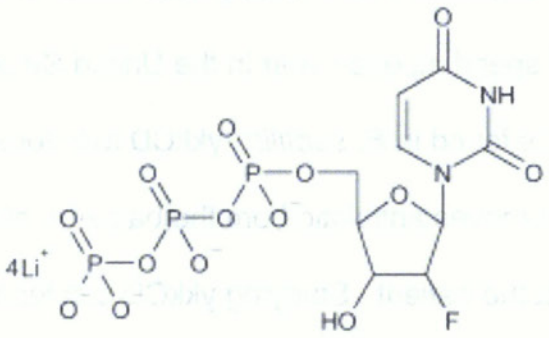

Figure 7 - Structure of 2'F-dUTP

The effectiveness of the modifications can be tested by incubating the RNA in fetal bovine serum, which contains high levels of ribonucleases. If the modifications are successful, then the RNA will degrade at a slower rate than the unmodified version. These modifications were chosen because the impact on 3D structure and binding is expected to be minimal (Bui 13). The structure of the ykkCD must remain relatively unchanged for it to retain its specificity for tetracycline.

Project Applications

The medical significance of this project arises from the fact that ykkCD is a tet-responsive regulator of resistance bacteria. If the underlying mechanism of antibiotic sensors like ykkCD can be understood, it will provide opportunities to suppress resistance mechanisms and innovate new nanotechnology applications.

Bacterial resistance is a growing problem in the medical field. According to the CDC, 70% of bacterial strains are resistant to at least one antibiotic. Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most common resistant strains and is responsible for 369,000 hospitalizations, 19,000 deaths, and $4 billion in health-related spending each year in the United States. The ykkCD riboswitch used in this project is found in *B. subtilis*. ykkCD functions to regulate expression of an efflux pump, which removes antibiotic from the bacteria, allowing it to resist tetracycline and continue to infect the patient. Studying ykkCD can lead to the development of new methods to treat bacterial infections that will bypass the efflux pump defense mechanism.

The nanotechnology applications of ykkCD include use as a highly sensitive tet sensing module or a tet-responsive regulatory switch. Short term goals include using ykkCD as a tet-delivery capsule to treat infections caused by tet-resistant bacteria. By caging tetracycline in the ykkCD aptamer, it may avoid recognition by the resistance mechanisms in bacteria. If the tetracycline avoids detection, then the drug can again be useful as a therapeutic. Long term goals include utilizing the highly sensitive binding site for various applications. Binding affinity measurements show that ykkCD has a binding affinity of 2.4nM, meaning that it can detect an extremely small amount of tet molecules within a *B. subtilis* cell. ykkCD can be modified to respond to man-made signals. It can also function as a tet-responsive molecular scissor that degrades harmful compounds in a tet-dependent manner. Finally, once the molecular basis of the extreme sensitivity is understood, the specificity of ykkCD can be altered and it can be used as a caging compound for targeted drug deliveries.

Research Objectives

This project focused on attaching ykkCD to well-characterized gold nanoparticles and working to improve the stability of the RNA in mammalian cell environments. The first stage in creating a medically useful conjugate is developing a method of attaching ykkCD to well-characterized nanoparticles. The RNA will be linked to the nanoparticles using a sulfydryl modified DNA oligonucleotide that is designed to be complementary to nucleotides 85-106 of ykkCD. This region of ykkCD is not believed to be functional in tet sensing, so the attachment of the oligonucleotide should not alter the specificity of the riboswitch. SH functionalized oligonucleotides have also been demonstrated to form stable interactions with gold nanoparticles. ykkCD is attached to the oligo through a heat annealing process that allows the bases of the riboswitch to form hydrogen bonds to the oligonucleotide. To test if the attachment to the nanoparticle is successful, several different methods can be used. First, a gel shift can show if the RNA and oligo complex has attached to the nanoparticle. If the RNA is successfully attached to the nanoparticle, it will increase the overall size of the particle. This will cause a sample of functionalized gold nanoparticles to move at a slower rate than nanoparticles alone. The size of the particles can also be measured using dynamic light scattering (DLS). If the attachment process is successful, the dynamic light scattering will show an increase in size between the naked gold nanoparticle and the nanoparticle conjugates. In addition to the gel shift and DLS, the attachment of the RNA to the nanoparticle will alter the UV-Vis spectra of the naked nanoparticle.

Project Goals

As we stated earlier, the riboswitch must be modified to protect it from ribonucleases and attached to well-characterized nanoparticles to increase bioavailability. This project focuses on modifying the ykkCD using dUTP bases and 2'-Fluoro RNA oligonucleotides. The stability of the modified RNA will be assessed by incubating the samples in fetal bovine serum. The level of degradation of the samples will indicate how if the modified RNA samples are more stable than the unmodified version. Then the resulting modified RNAs will be attached to gold nanoparticles (Figure 8). The conjugates will be purified and used in functional tests.

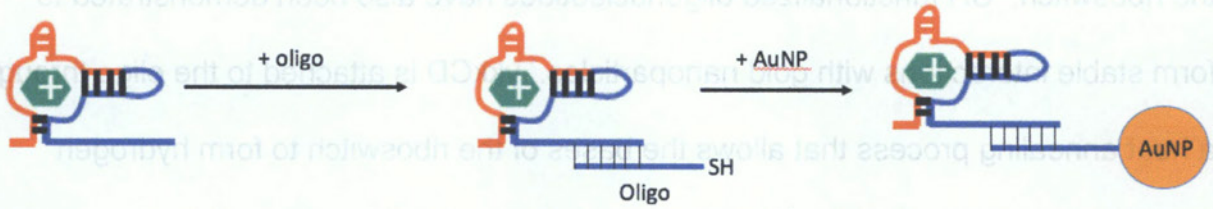

Figure 8 - Schematic of the attachment of ykkCD and AuNP (green hexagon represents tetracycline)

Materials and Methods

Section 1: DNA and RNA synthesis

Large scale PCR to make ykkCD riboswitch template DNA

A polymerase chain reaction (PCR) technique was used to create large quantities of ykkCD riboswitch templates. The components listed in table 1 were combined and mixed in a 1.5 mL centrifuge tube. 100 μL aliquots were transferred into 10 separate 0.5 mL microcentrifuge tubes, which were then placed into the PCR

machine. The PCR program listed in table 2 was used to amplify the DNA and create ykkCD riboswitch templates. Following completion of the program, an IBI Scientific PCR cleanup kit was used to purify the resulting riboswitch templates according to the instructions of the manufacturer.

Table 1. *Components for a 1mL PCR reaction*

1 μL ykkCD riboswitch DNA template
10 μL 100 μM primer (top)
10 μL 100 μM primer (bottom)
200 μL 5x reaction buffer
20 μL dNTP mixture
739 μL millipore water
20 μL DNA polymerase

Table 2. *PCR program details*

	Temperature	Time	Function
Step 1	98°C	30 minutes	
Step 2	98°C	5 minutes	Separate DNA strands
Step 3	39°C	5 minutes	Anneal primers
Step 4	72°C	15 minutes	Amplification of DNA
Repeat steps 1-4 for 35 cycles			
Step 5	72°C	1 minute	Final amplification
Step 6	4°C	Overnight	Storage

RNA synthesis using PCR DNA templates

Large amounts of ykkCD RNA were produced using the DNA templates and T7 RNA polymerase. The reagents listed in table 3 were combined into a 1.5mL microcentrifuge tube. The reaction mixture was placed into a 37°C water bath for two hours. After two hours, 10μL of RNase free DNase was added to eliminate any remaining DNA templates. The reaction mixture was incubated for an additional 30 minutes. After 30 minutes, 50μL of EDTA was added. The concentration of the RNA was measured using UV spectroscopy. This protocol typically produced RNA samples with a concentration of 150-200 ng/μL. Samples were stored at -20°C until needed.

Table 3. *Reagents for a 500 μL transcription reaction mixture*

50 μL 10x reaction buffer
10 μL 10 mM rNTP mixture
10 μg DNA (from purified PCR product – volume varies with concentration)
25 μL100mM DTT
50 μL T7 Polymerase (Epicentre, catalog no. TH950K)
Add Millipore H_2O until reaction mixture reaches 500 μL

Synthesis of dUTP modified ykkCD RNA

The ykkCD RNA was prepared using dUTP instead of rUTP to increase its stability. The reagents in table 4 were combined according to the instructions provided by the manufacturer in a 1.5mL microcentrifuge tube. The polymerase used in this synthesis was an Epicentre T7 R&DNA™ polymerase, which was chosen due to its

ability to incorporate modified bases into the transcription products. The tube was incubated in a 37°C water bath overnight. After the incubation period, the dUTP RNA was purified using either standard column purification or gel purification protocol. Following the purification protocol, the concentration of the dUTP RNA sample was checked using UV spectrometry. This protocol typically produced samples of dUTP RNA with a concentration of 5-25 $\mu g/\mu L$.

Table 4 - *Reagents for a 500 μL dUTP RNA transcription reaction mixture*

50μL 10X reaction buffer (provided by manufacturer)
25 μL 10 mM dUTP
25 μL 10 mM rATP
25 μL 10 mM rGTP
25 μL 10 nM rCTP
2.5 μL 1M $MnCl_2$
2.5 μg DNA template
25 μL 100 mM DTT
50 μL T7 R&DNA^{TM} Polymerase (Epicentre, catalog no. D7P9205K)
Millipore H_2O to 500 μL

Synthesis of 2'F modified ykkCD RNA

The ykkCD RNA was prepared using 2'F rUTP and 2'F rCTP to increase its stability. The reagents in table 5 were combined according to instructions provided by the manufacturer in a 1.5mL microcentrifuge tube. The polymerase used in this

synthesis was an Epicentre T7 R&DNA™ polymerase, which was chosen due to its ability to incorporate modified bases into the transcription products. The tube was incubated in a 37°C water bath overnight. After the incubation period, 10μL RNA-free DNase was added to the tube and then it was incubated for an additional 30 minutes at 37°C. Next, 100μL of 0.25M EDTA (pH=8) was added. The 2'F RNA was purified using either standard column purification or gel purification. The concentration of the sample was then measured using UV spectrometry.

Table 5 - *Reagents for a 500 μL 2'F RNA transcription reaction mixture*

50 μL 10X reaction buffer (provided by manufacturer)
5 μL 50mM 2'F dUTP
5 μL 50mM 2'F CTP
5 μL 10mM rGTP
5 μL10mM rATP
2.5 μL 1M $MnCl_2$
2.5 μg DNA template
25 μL 100mM DTT
50 μL T7 R&DNA™ Polymerase (Epicentre, catalog no. D7P9205K)
Millipore H_2O to 500 μL

Section 2: DNA and RNA purification

Purifying PCR Products using column purification

The DNA templates produced using PCR were purified using an IBI Scientific Gel/PCR DNA Fragments Extraction Kit (Cat. No. IB47020). The samples were purified

using the protocol outlined by the manufacturer. After purification, the concentration of the template samples was checked using UV spectrometry. After this process, the riboswitch templates were stored at -20°C until needed for production of ykkCD RNA samples. This protocol typically produced purified DNA samples with a concentration of approximately 150 ng/μL.

RNA purification using column purification

The transcription products were purified to remove any contamination from the RNA samples, including low molecular weight degradation products. Thermo Scientific GenJet RNA purification columns and the accompanying manufacturer instructions were used to purify the RNA. The concentration of the RNA was determined using UV spectrometry. The absorbance of the sample at 260 nm was multiplied by the specific molar extinction coefficient to find the concentration of the RNA sample. The specific molar extinction coefficient for the ykkCD RNA is 1149800.

Check RNA purity using polyacrylamide gel electrophoresis

To ensure that the RNA sample is free of degradation products, the samples were resolved using denaturing polyacrylamide gel electrophoresis (PAGE). A 20% denaturing urea gel and urea dye were prepared using standard protocols. Prior to loading the samples, the gel was pre-run for 30 minutes at 15W using a 0.5X TBE running buffer. The samples were prepared by adding 5μL urea dye to 5μL RNA. The sample was boiled on a 100°C heat block for 5 minutes and then were spun for 30

seconds on the centrifuge. After the gel pre-run was complete, the RNA samples were loaded into the wells. The gel was then run at 15W for 30 minutes.

After the electrophoresis step, the gel was removed from the glass plates and placed into a plastic container filled with 100mL 0.5x TBE. 10μL ethidium bromide was added to the container; it was placed on an orbital shaker for 10 minutes to stain the gel. Finally, the gel was photographed using the Bio-Rad gel imager. The RNA sample is pure if there is a single band on the gel. If there are multiple bands, that indicates that there are other types of DNA or RNA in the mixture.

Gel purification of RNA products

Gel purification was used to separate full length RNA from its degradation products; the full-length RNA was excised and eluted from the gel.

Sample Preparation

To prepare the samples for gel purification, the RNA was precipitated using a salt/ethanol mix and dried using the speed vac. After drying, the RNA was combined with urea dye. The sample was resolved on a 20% denaturing gel, which was run for 1 hour at 15W.

Gel Excision

First, the urea gel was removed from the glass plates and placed on clean plastic wrap. The gel was then placed above fluorescent paper and a UV light was directed onto the sample, showing the full length RNA as a dark line. The band of RNA was cut

from the gel using a sterilized razor blade. The gel excision was placed into a syringe and was crushed into a 2mL centrifuge tube.

RNA Elution

After the gel excision, elution buffer was used to elute the RNA from the crushed gel. 1 mL of 1X TBE (tris borate, EDTA pH = 8.0) elution buffer and 1 mL of phenol/cholorform was added to the crushed gel. The sample was incubated at 37°C for 2 hours. Following incubation, the sample was centrifuged at maximum speed for 2 minutes. The top layer of the sample was transferred to a clean 2 mL centrifuge tube, which was then placed on ice. An additional 2 mL of elution buffer was added to the original tube. The sample was incubated for an additional 2 hours at 37°C. After the second incubation, the sample was centrifuged at maximum speed for 2 minutes. The top layer of the sample was placed into a clean 2 mL tube, which was then placed on ice.

Ethanol precipitation of elution fractions

Ethanol precipitation was used to collect the RNA from the elution fractions. 500μL of chloroform was added to each of the elution fractions. Each tube was vortexed for 1 minute and then centrifuged for 2 minutes. The top layer of each fraction was transferred to a new 2 mL tube. The following components were added to each of the 2mL tubes:

- 100μL 3M Na-acetate (pH=5.3)
- 1mL isopropanol
- 1μL glycogen

Next, the samples were incubated at -20°C overnight.

RNA precipitation

After the samples were incubated overnight, they were allowed to thaw and then were centrifuged for 30 minutes at maximum speed. The supernatant was removed and 100μL of cold 75% EtOH was added to the tube. The samples were then centrifuged for 5 mintues at maximum speed and then the supernatant was removed. The pellet was then dried in the speed vac for 5 minutes and then resuspended in 100μL TE.

Gel purified RNA purity check

After the RNA was precipitated, its purity was checked using a urea gel. The gel was prepared using the same protocol as outlined above. 5μL of the purified RNA was loaded into the well and the gel was run for 30 minutes at 15W. The gel was then removed from the glass plates and placed into a plastic container with 100mL 0.5X TBE and 10μL Ethidium Bromide. The container was placed on a mutator for 5-10 minutes and then photographed using the BioRad gel imager system.

Section 3: AuNP synthesis and purification

Synthesis of Aqueous Citrate-Protected Gold Colloid

The Tanton protocol was followed to perform a simple synthesis of aqueous gold nanoparticles surrounded by shells of coordinated citrate anions (Tanton 6). In this

protocol, sodium tetracholoraurate ($NaAuCl_4$) is reduced by a citrate salt to yield particles approximately 15 nm in diameter.

Materials:

- Aqua regia: 3:1 (v/v) concentrated HCl/concentrated HNO_3
- 1 mM $HAuCl_4$
- 38.8 mM sodium citrate
- 500 mL round-bottom flask
- Reflux condenser
- Heating mantle
- 0.45-μm nylon filter

Procedure:

First, all of the glassware involved in the process was washed thoroughly with aqua regia and then with water. After washing, the glassware was assembled on a heating mantle and magnetic stirrer. A large stir-bar was added to the 500mL round-bottom flask. The flask was charged with 250 mL of 1 mM $NaAuCl_4$ and the solution was brought to reflux with vigorous stirring. Next, 25mL of 38.8 mM sodium citrate was added to the round bottom flask. The sodium citrate was added as rapidly as possible. The addition of the sodium citrate resulted in the solution changing from a yellow to purple color. The solution remained in reflux for 20 minutes. By the end of the reflux period, the solution was a deep red color. The solution was then cooled to room

temperature and filtered through a 0.45 μm nylon filter. The nanoparticle solution was stored at room temperature in a dark glass container.

Section 4: Conjugation and Purification of AuNPs

Preparation of Gold Nanoparticle-DNA conjugates

Materials:

- oligonucleotide dissolved in water
- aqueous gold nanoparticle solution
- Bis(p-sulfonatophenyl)phenyl-phosphine dehydrate dipotassium salt (Aldrich)
    - Solid and 0.5 M aqueous solution
- NaCl, solid and 1M aqueous solution
- Methanol
- 5x TBE electrophoresis buffer
- 30% (v/v) glycerol

Procedure:

Complex nanoparticles with phosphine

First, 2 mg phosphine was added to 10 mL gold nanoparticle solution. Three separate types of nanoparticles were used: nanoparticles that were produced in-house using the Tanton protocol, PELCO NanoXact 15nm AuNPs (Product No. 82150-15), and PELCO 5nm gold colloid (Product No. 15702-20). This solution was rotated for 10 hours at room temperature on an orbital shaker at low speed. After 10 hours, solid NaCl was added to the tube to create a solution of approximately 2-3M NaCl, resulting in a color

change from deep burgundy to light purple. The particle solution was then centrifuged for 30 minutes at 500xg at room temperature to pellet the particles. After centrifugation, the supernatant was discarded and the particles were resuspended in 1 mL of 0.5 mM phosphine. 0.5 mL methanol was added to the particle solution. The solution was again centrifuged for 30 minutes at 500 x g at room temperature. After this centrifugation step, the supernatant was discarded and the nanoparticles were resuspended in 1 mL of 0.5 mM phosphine. 110 μL 5x TBE electrophoresis buffer was added to result in a final concentration of 0.5x TBE. The absorbance of the nanoparticle solution was measured using the nanodrop spectrometer. To find the concentration in molarity, the absorbance of the sample was multiplied by the extinction coefficient for the nanoparticles. 15nm nanoparticles have an extinction coefficient of 6.5×10^8; 5nm nanoparticles have an extinction coefficient of 4.5×10^7.

Heat anneal oligonucleotide and ykkCD RNA

Equal concentrations of deprotected oligonucleotide and ykkCD RNA were combined in a microcentrifuge tube. The tube was heated at 100°C for 2 minutes. After heating, the tube was spun in the centrifuge for 30 seconds and then placed on ice for 10 minutes. Finally, 55μL 1M NaCl was added to the solution before adding the solution to the nanoparticles.

Conjugate nanoparticles and oligonucleotide

First, an aliquot of oligonucleotide-ykkCD RNA solution was diluted in enough 0.5x TBE to make a final solution of 50μM oligonucleotide. The oligonucleotide and nanoparticle solutions were then combined into a tube and mixed well. 0.05 vol of 1M NaCl was added to the tube; the tube was then placed on an orbital shaker at low speed and allowed to incubate for 16 hours.

Purification of conjugates

The 5nm conjugates were resolved on a 1% agarose gel. Bands belonging to the conjugate were excised per the Tanton protocol. ykkCD-AuNP conjugates were recovered from the gel using electroelution. Finally, the absorbance of the sample was checked using UV spectrometry. The 15nm commercial AuNP conjugates were purified using a sequence of four 30-minute centrifugations. The conjugates were centrifuged; the supernatant was discarded and then the samples were resuspended in 100μL 0.5X TBE. After the centrifugations, these conjugates were also analyzed using UV spectrometry.

Section 5: End Point Stability Test

Blood Serum Test – End-point Assay

The blood serum test was used to compare the stability of unmodified ykkCD RNA and derivativized ykkCD RNA samples. The samples were prepared as follows:

Unmodified RNA:

- 1μL RNA
- 1μL 10% Fetal Bovine Serum or H_2O (Control)
- 2 μL 5x reaction buffer
- 6 μL H_2O

Total Volume = 10 μL

dUTP RNA:

- 5 μL dUTP RNA
- 1 μL 10% Fetal Bovine Serum or H_2O (Control)
- 2 μL 5x reaction buffer
- 2 μL H_2O

Total Volume = 10 μL

2'F RNA:

- 5 μL 2'F RNA
- 1 μL 10% Fetal Bovine Serum or H_2O (Control)
- 2 μL 5x reaction buffer
- 2 μL H_2O

Total Volume = 10 μL

After the reagents were combined, the samples were incubated for 1 hour in a 37°C water bath. 10 μL urea dye was added and the samples were placed on a heating block for 5 minutes at 100°C. After heating, the samples were resolved on a 10% denaturing gel. The gel was then placed in a container with 100mL 0.5x TBE and 10μL Ethidium

bromide and placed on a mutator for 10 minutes. After staining, the gel was photographed using the BioRad gel imaging system.

Determining the Time-Dependence of RNA Degradation

Fetal bovine serum and ykkCD RNA samples were incubated in a 37°C water bath; 5 μL samples removed at time 0, 10, 20, 25, 40, 60 and 80 minutes. A control of ykkCD RNA mixed with water was left in the water bath for the full 80 minutes. The starting reaction volume was 40 μL and was composed of the following reagents were combined in a 1.5 mL microcentrifuge tube:

- 10μL ykkCD RNA
- 8 μL 5x reaction buffer
- 4 μL 10% Fetal Bovine Serum
- 18 μL H_2O

After the reagents were mixed, a 5 μL sample was immediately removed (time = 0). The tube was placed in the water bath until 10 minutes had elapsed. At this point, an additional 5μL sample was removed from the reaction and was placed in a separate tube with 5 μL urea dye. The sampling procedure was repeated after 20, 25, 40, 60 and 80 minutes had elapsed. Each 5μL RNA sample was placed into a clean microcentrifuge tube and mixed with 5μL urea dye. After mixing with the urea dye, the samples were placed on a 100°C heating block for 5 minutes and then were stored on ice until all samples had completed the process. After all the samples were removed and mixed with the urea dye, they were loaded into wells of a urea gel. The samples

were then run at 15W until the blue urea dye reached the bottom of the gels. The gel was then removed from the glass plates and placed in a container with 100μL 0.5x TBE and 10μL EtBr. The gel was placed on a mutator for 10 minutes and then photographed with the BioRad gel imaging system. The volume of the bands was quantified using the ImageLab software program. This stability test was repeated for unmodified ykkCD RNA, dUTP modified RNA, and 2'F modified RNA.

Section 6: Attachment Analysis

Gel shifts, dynamic light scattering, and UV-Vis spectroscopy were used to test if the nanoparticles and RNA had successfully conjugated.

Gel Shift Assay to Demonstrate RNA-AuNP Attachment

If the attachment was successful, the AuNP conjugates would move more slowly than naked nanoparticles through an agarose gel. Multiple lanes of a 2% agarose gel were loaded with conjugate samples and were run at 150V for 30 minutes. The first well was filled with 20μL naked AuNP and 4μL 30% glycerol. The next well was filled with 20μL dUTP + AuNP conjugate and 4μL 30% glycerol. The third well was filled with 20μL 2'F + AuNP conjugates and 4μL 30% glycerol. Gel shifts were also performed with the naked AuNP and the conjugate samples combined in a single well. In this case, 10μL naked AuNP, 10μL AuNP conjugates, and 4μL 30% glycerol were combined and then loaded into the agarose gel. After the samples were run on the gel, the gel was photographed using the BioRad gel imaging system and the movement of the bands was compared.

Dynamic Light Scattering

Successful conjugation of the RNA to the nanoparticle results in an increase in size. This size change can be detected using dynamic light scattering. Three different samples were tested:

- Naked AuNP
- AuNP + dUTP RNA
- AuNP + 2'F RNA

Individually, 100μL of each sample was loaded into a glass cuvette and were placed into a Malvern Zetasizer nano DLS machine. The data was recorded and exported to Microsoft Excel for analysis.

UV-Vis spectroscopy

Attachment of the RNA to the nanoparticle would also result in changes in the sample's UV-Vis spectrum. Three separate samples were tested:

- Naked AuNP
- AuNP + dUTP RNA
- AuNP + 2'F RNA

Individually, the samples were loaded into a glass cuvette and were placed into a HP 8452A Diode Array Spectrophotometer for UV-Vis analysis. The samples were tested in a wavelength range from 190 to 820 nm. The spectra data was then exported to Microsoft Excel for analysis.

Results

Stability Tests

The dUTP and 2'F modifications to ykkCD were tested for increased stability in fetal bovine serum. After incubation with the fetal bovine serum, the samples were loaded onto denaturing gels so that the fraction of intact RNA could be determined. The unmodified RNA showed virtually no intact RNA after the first minute. The RNA continued to degrade further over the remainder of the incubation period. Both modified RNA samples showed much higher percentages of intact RNA when incubated with the same concentration of fetal bovine serum. The fraction of intact RNA remaining after each incubation period is represented in Figure 9-12.

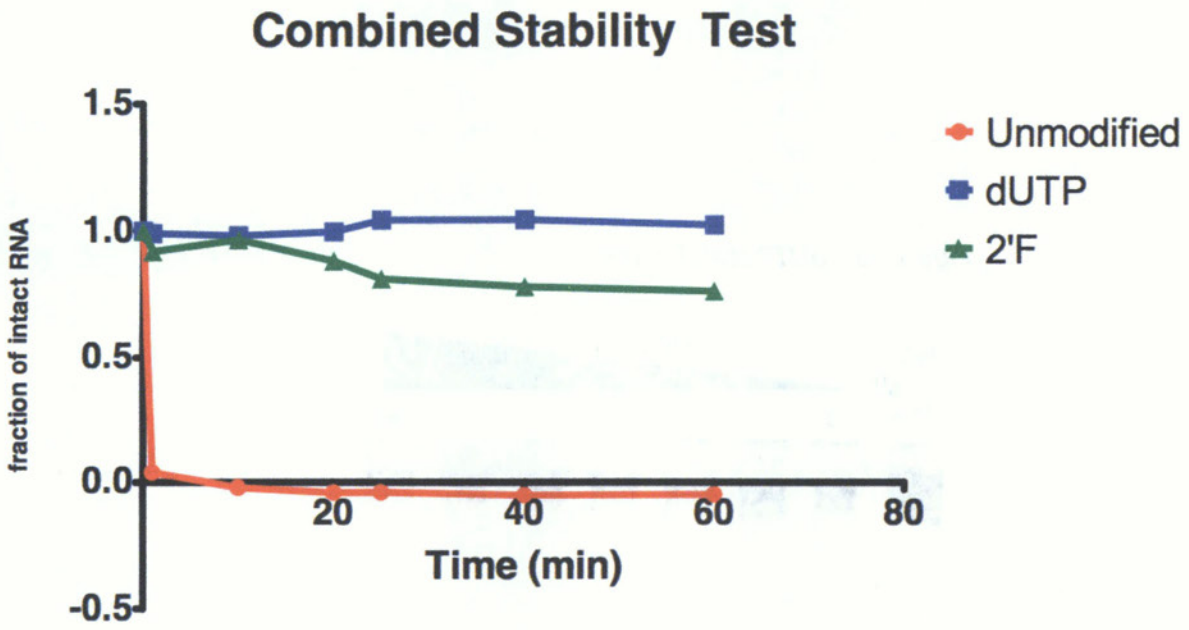

Figure 9 - Fraction of intact RNA remaining following incubation with FBS

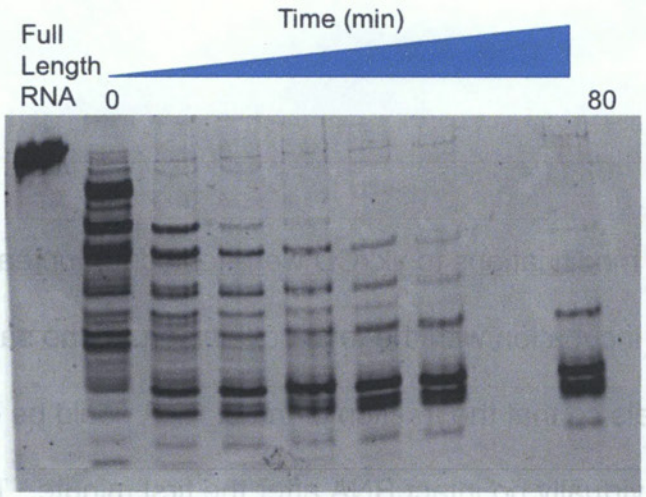

Figure 10 - Unmodified RNA stability test

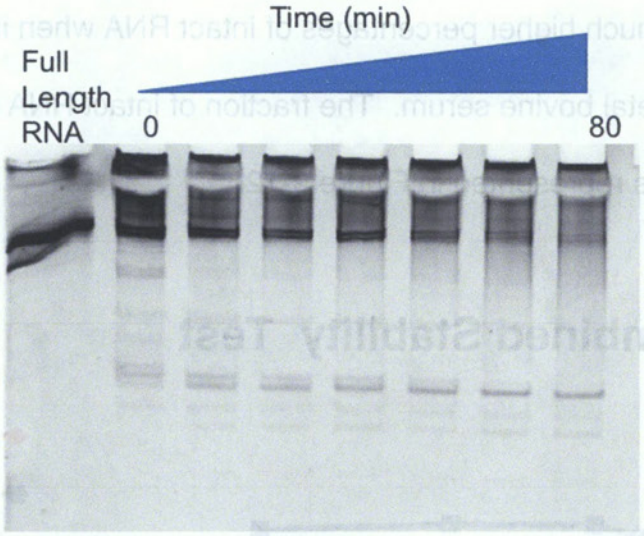

Figure 11 - dUTP stability test

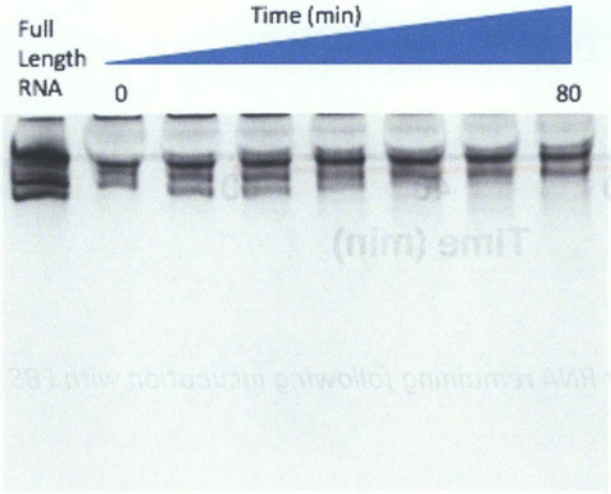

Figure 12 - 2'F Stability Test

Purification Methods of RNA-AuNP conjugates

The purification of the RNA-AuNP conjugates was adjusted based on the size of the particle. The purification of the 5nm AuNP was most successful when it was resolved on a 1% agarose gel. The increase in the size between the naked AuNP and the conjugate was significant enough to create a shift on the gel. Thus, the conjugate could easily be separated from the naked particle. The change in size between the naked 15 nm AuNP and the 15nm conjugate was not significant enough to create an easily visible shift on an agarose gel. The best way to purify the 15nm particles was through a series of centrifugations and resuspensions.

Evidence for Successful Attachment

The ykkCD was successfully attached to the nanoparticles; this was shown through gel shifts, dynamic light scattering, and UV-Vis spectroscopy. When naked AuNPs and AuNP conjugates were resolved on a 2% agarose gel, there is a clear shift between the bands. As shown in figure 12, The bands with the dUTP and 2'F RNA

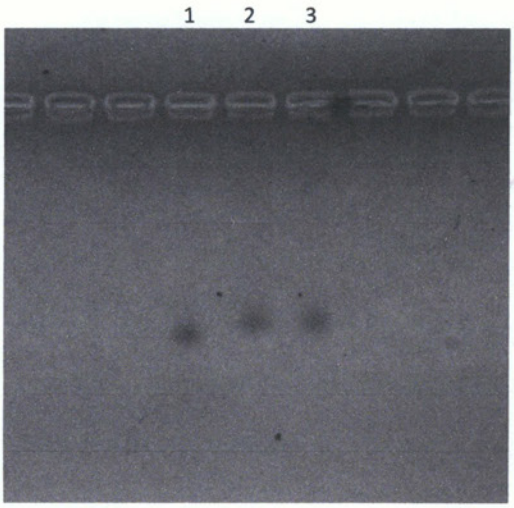

Figure 13 - Gel shift between naked AuNP and conjugates on a 2% agarose gel

Lane 1 – Naked AuNP
Lane 2 – 2'F RNA + AuNP
Lane 3 – dUTP RNA + AuNP

attached to the nanoparticle moved a shorter distance through the gel than the nanoparticle alone.

The sizes of the nanoparticles and the conjugates were compared using dynamic light scattering. There was an increase in size between the naked nanoparticle and the RNA conjugates. In the 15nm test, the conjugates with the 2'F RNA attached showed the largest increase in size (Figure 14). The dUTP RNA also increased the size of the conjugate, but it was a smaller change than the 2'F. The 5nm particles also showed an increase in size after the attachment of RNA. In the 5nm samples, the largest increase in size was caused by the addition of the dUTP (Figure 15). The addition of 2'F created a small increase in conjugate size.

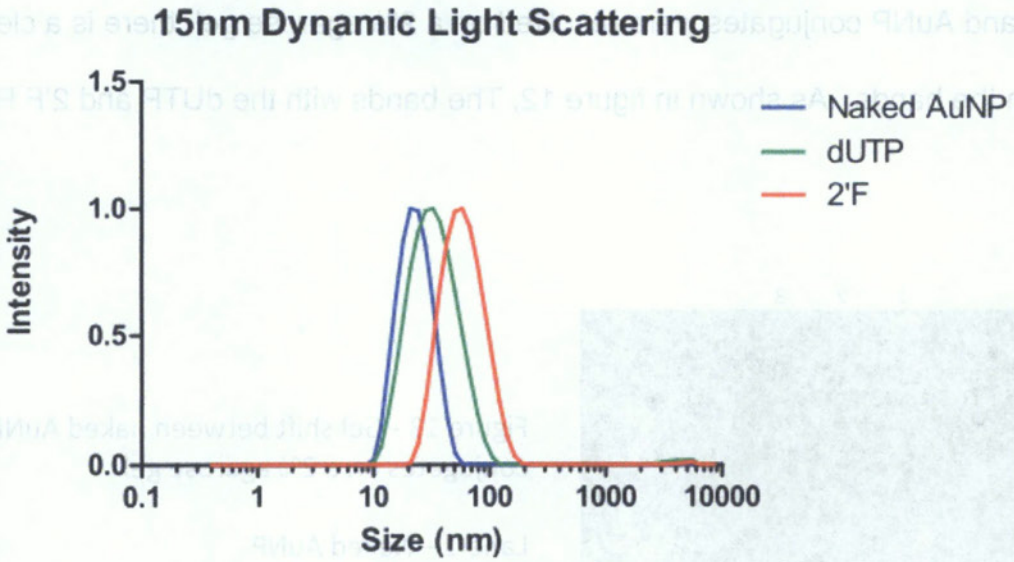

Figure 14 - Dynamic light scattering size comparison of 15nm AuNPs and conjugates

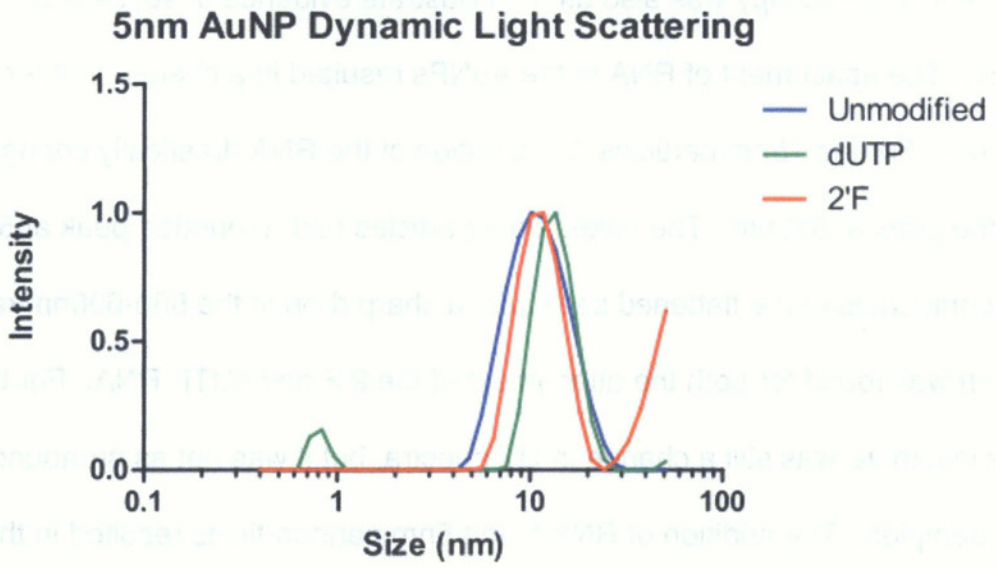

Figure 15 - Dynamic light scattering size comparison of 5nm AuNPs and conjugates

Table 6 - Average conjugate size

Sample	Average Size	Standard Deviation
15nm - Naked	23.29	7.038
15nm - dUTP	35.21	16.14
15nm - 2'F	60.74	22.87
5nm - Naked	11.44	3.918
5nm - dUTP	12.18	2.432
5nm - 2'F	11.83	2.903

UV-Vis spectroscopy was also used to illustrate evidence of successful attachment. The attachment of RNA to the AuNPs resulted in a change in the sample's UV spectrum. For the 15nm particles, the addition of the RNA drastically changed the shape of the peak at 520nm. The naked nanoparticles had a rounded peak at 520nm, while the conjugates had a flattened peak with a sharp drop in the 580-600nm range. This pattern was found for both the attachment of the 2'F and dUTP RNA. For the 5nm nanoparticles, there was still a change in UV spectra, but it was not as pronounced as in the 15nm samples. The addition of RNA to the 5nm nanoparticles resulted in the peak at 520nm becoming less sharp and increasing the width of the peak. The introduction of the RNA also increased the sample's absorbance in the 600-800nm range. The spectra for the 2'F and dUTP are again similar, but for the 5nm samples, the peak for the dUTP is slightly sharper than the 2'F. (Fig. 16-19)

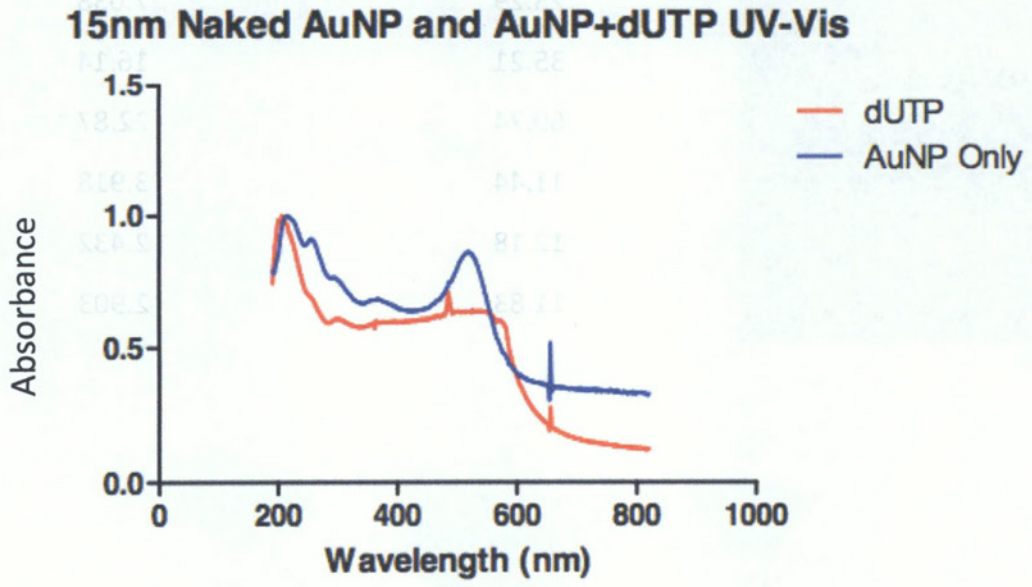

Figure 16 - UV-Vis spectrum comparison of 15nm AuNPs and dUTP conjugates

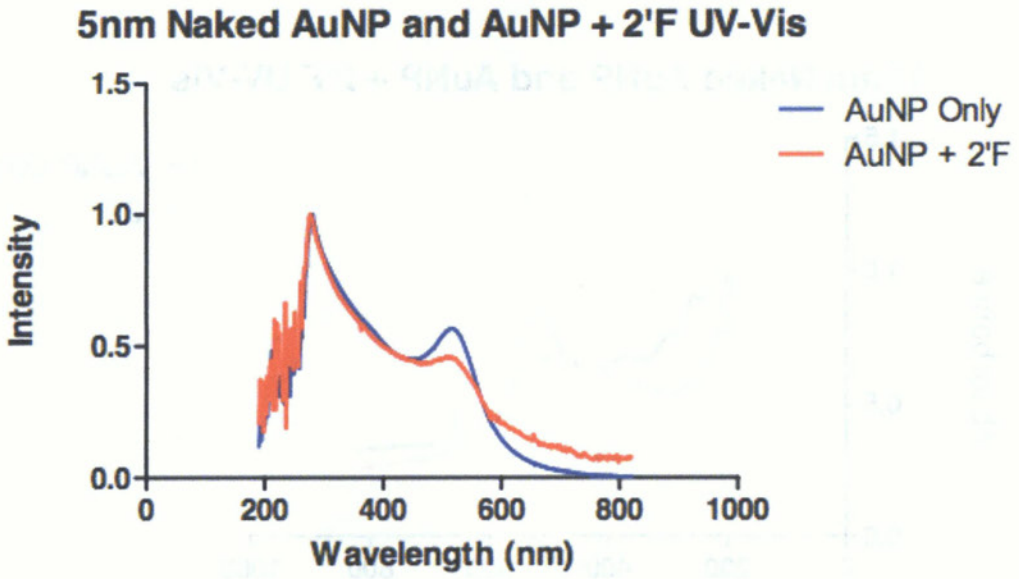

Figure 19 - UV-Vis spectrum comparison of 5nm AuNPs and dUTP conjugates

Discussion

In this project, the ykkCD riboswitch was modified to increase its stability and then the riboswitch was attached to well-characterized gold nanoparticles. The two separate modifications to ykkCD included substituting deoxy UTP for the natural UTP base in some samples and replacing pyrimidine nucleotides with 2'Fluoro derivatives in others. The RNA was then attached to gold nanoparticles using a linker oligonucleotide. To determine if the attachment was successful, the samples were analyzed using gel shifts, dynamic light scattering, and UV spectroscopy.

Stability Tests

To determine if the dUTP and 2'F modifications increased the stability of the RNA, several stability tests were performed. The RNA was incubated with fetal bovine serum and samples were taken at specific time intervals. The samples and a control

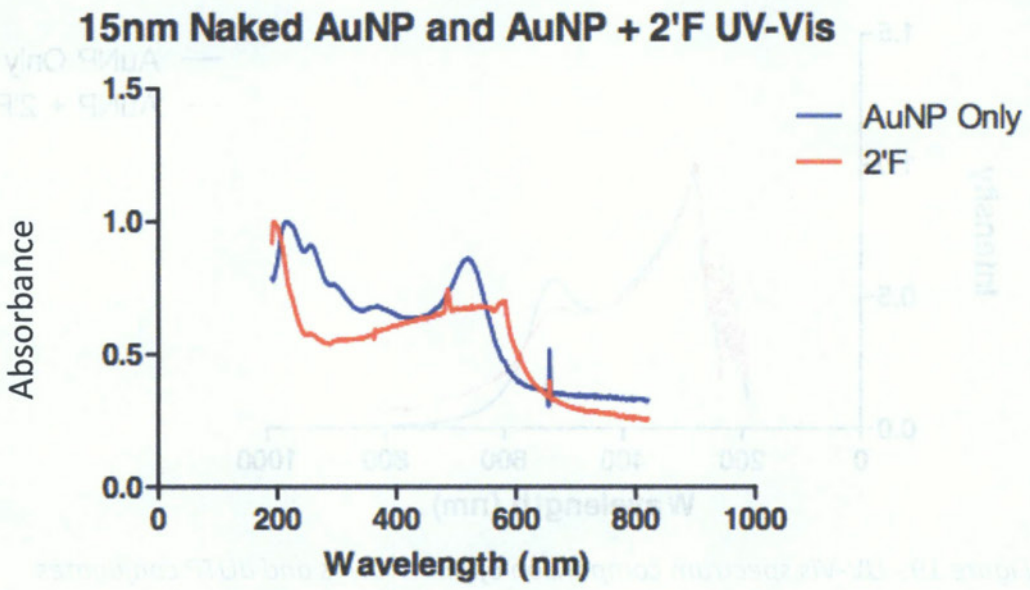

Figure 17 - UV-Vis spectrum comparison of 15nm AuNPs and 2'F conjugates

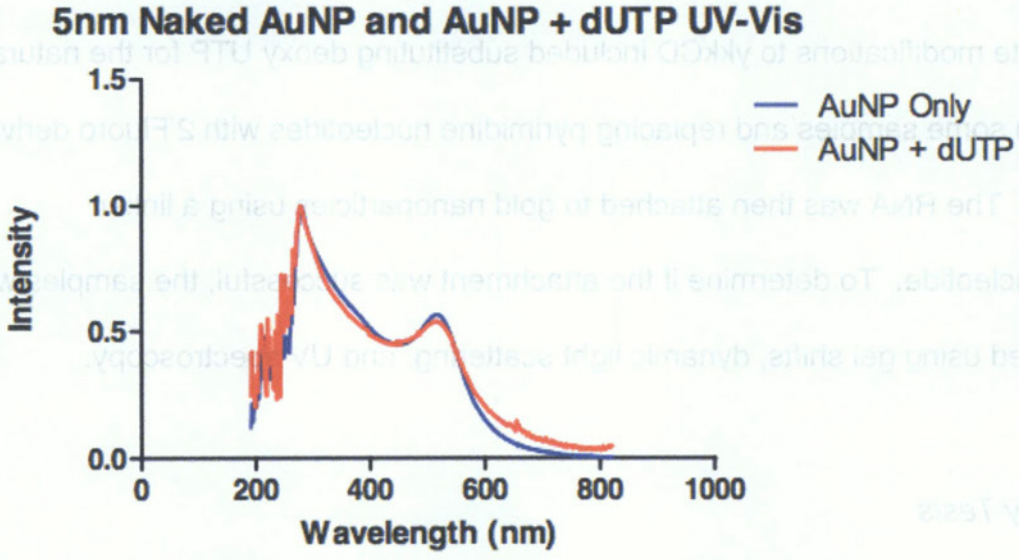

Figure 18 - UV-Vis spectrum comparison of 5nm AuNPs and dUTP conjugates

(no FBS) were resolved on a urea gel, the arrangement of the bands gave an indication of the stability of the RNA. If the RNA remained intact, then the samples would show a band the same size and position as the control. If the RNA was degraded by the ribonucleases in the FBS, then there would be smaller bands that migrated further down the gel. This shows that the overall RNA structure was broken down into smaller pieces, which moved through the gel faster than the larger control sample. A visual inspection of the unmodified RNA shows that the sample was degraded almost completely after the first incubation period. The sample is broken up into many bands which migrated a significant distance down the gel, indicating a small size. Both the dUTP and 2'F modified samples show a much higher percentage of intact RNA throughout the duration of the incubation periods. The dUTP modified samples showed a small amount of degradation in the form of several faint bands that migrated down the gel. The 2'F modified samples displayed a very similar band pattern as the control sample.

The results of the stability test were quantified using Bio-Rad ImageLab software. The volume of the band corresponding to the intact RNA was quantified and then a background measurement was subtracted from the measurement. The data was also controlled for the total amount of RNA loaded into each well. Next, the volume for each incubation period was divided by the volume of the control sample. The resulting data was then plotted in Figure 15. The chart illustrates the fraction of intact RNA over time. The unmodified RNA shows a very small fraction of intact RNA after the first incubation period. Both the dUTP and 2'F modified RNA displayed a much higher level of intact

RNA over the duration of the experiment. This indicates that the modifications made to the RNA were successful in protecting the structure from degradation by ribonucleases. This stability is critical if ykkCD is to function properly as a human therapeutic tool. If the RNA is not stable in the presence of ribonucleases, then it will be degraded soon after it is introduced to a mammalian environment and cannot work as a therapeutic tool.

Attachment Analysis

For ykkCD to be successfully used in human tissues, it is critical that the RNA remains bioavailable. Attachment of the RNA to the nanoparticles allows the complex to be engulfed by cells. The RNA was first attached to a linker oligonucleotide via a heat annealing process. The oligonucleotide was then connected to the nanoparticles through the thiol-gold affinity interactions. The attachment of the RNA to the nanoparticles was successful, and this was shown in three different ways.

Gel shifts were used to determine if the RNA had successfully attached to the AuNPs. If the attachment was successful, then the conjugates will move through an agarose gel more slowly than just the nanoparticles alone. In this experiment, samples were resolved on a 2% agarose gel. Both the dUTP modified RNA and the 2'F modified RNA caused the conjugate samples to move through the gel more slowly than the nanoparticles alone. This indicates that the RNA was securely attached to the nanoparticle and the objective of the study was met.

Attachment of the RNA was also analyzed using dynamic light scattering (DLS). Dynamic light scattering is a process that assesses the size of particles by directing a light source at a solution and analyzing the intensity and the pattern of light that scatters after hitting the particles. If the attachment is successful, then a comparison of the naked nanoparticle and a RNA conjugate will show an increase in size. The dynamic light scattering test also indicated that the attachment process was successful for both types of nanoparticle. The 15nm nanoparticle tests showed that the attachment of the RNA significantly increased the size of the particles. The addition of the 2'F RNA increased the average size from 23.29 ± 7.038 nm to 60.74 ± 22.87 nm. The addition of the dUTP RNA increased the average size from 23.29 ± 7.038 nm to 35.21 ± 16.14 nm assuming that the structures were spherical in shape. The 5nm commercially produced nanoparticles measured 11.44 ± 3.918 nm using DLS. When the 2'F RNA was attached, there was a slight size increase from 11.44 ± 3.918 nm to 11.83 ± 2.903 nm. The dUTP RNA also increased the size of the conjugates slightly, from 11.44 ± 3.918 nm to 12.18 ± 2.432 nm. The increases in size shows that the RNA was successfully attached to the gold nanoparticles.

The final way that the attachment of the nanoparticles was analyzed was using UV-Vis spectroscopy. If the RNA was successfully attached, it would cause a change in the UV-Vis spectra of the sample. The naked AuNP was expected to have a well-defined peak at 520 nm. For the 15nm particles, the addition of the 2'F and dUTP RNA caused distinct changes to the UV-Vis spectra. The conjugates did not have a defined peak at 520nm, instead there was a sharp drop-off in the 580-600nm range. The naked

AuNP and the conjugates also had different absorbances in the 200-400nm range. The naked AuNP samples consistently had significantly higher absorbances throughout this range. These distinct changes indicate that the RNA was successfully attached to the gold nanoparticles. The 5nm samples also displayed changes in the UV-Vis spectra, but they were not as distinct as those shown in the 15nm samples. The dUTP conjugates showed a distinct peak at 520nm, but the peak was spread over a larger range of wavelengths. The sample showed an increase in absorbance in the 600-800nm range. The 2'F conjugates also displayed a wider peak at 520nm and an increase in absorbance in the 600-800nm range. The changes in the UV-Vis spectra again suggest that the attachment of the RNA to the nanoparticle was successful.

The overall goals for this study were to increase the stability of the ykkCD riboswitch and to show that it can be successfully attached to well-characterized gold nanoparticles. Both objectives were met. The dUTP and 2'F modifications were effective in increasing the stability of ykkCD in the presence of ribonucleases. The increased stability was visualized by comparing the fraction of intact RNA over time. The attachment of the RNA to the nanoparticles was also successful and was shown several different ways. The gel shifts, dynamic light scattering, and the UV-Vis analysis all showed that the RNA had been successfully attached to the AuNPs. The results of this study will help future researchers develop a method of using ykkCD to treat resistant bacteria and to eventually use the unique properties of the riboswitch to perform other advanced therapeutic processes.

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